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14. ABSTRACT
One in 8 women in the United States will develop breast cancer during her lifetime. Deaths are due to tumors that have metastasized. Photodynamic therapy (PDT) is a promising cancer treatment in which a photosensitizer (PS) accumulates in tumors and is subsequently activated by visible light of an appropriate wavelength. PDT produces cell death and tumor ablation. Mechanisms include cytotoxicity to tumor cells, shutting down of the tumor vasculature, and the induction of a host immune response. Mechanisms involved in the PDT-mediated induction of anti-tumor immunity are not yet understood. Potential contributing factors are alterations in the tumor microenvironment via stimulation of proinflammatory cytokines and direct effects of PDT on the tumor that increase immunogenicity. We have studied PDT of 410.4 variant 4T1 tumors growing in the mammary fat pad (orthotopic) in Balb/c mice and which produce metastasis. We have shown that a PDT regimen that produces vascular shutdown and tumor necrosis leads to initial tumor ablation but the tumors recur at the periphery. We studied the combination of PDT with immunostimulating therapies. Low dose cyclophosphamide is a mechanism to deplete regulatory T cells; these cells play a role in the immunosuppression activity of tumors. In combination with PDT, cyclophosphamide increases the survival. The second alternative therapy is the use of a novel combination of the immunostimulant CpG oligodeoxynucleotides (CpG-ODN) and PDT. CpG-ODN directly or indirectly triggers B cells, NK cells, macrophages and dendritic cells to proliferate, mature and secrete cytokines, chemokines and immunoglobulins. Both these novel combinations gave significantly enhanced therapeutic benefit not seen with single treatments alone. We propose that a rational choice of immune stimulant is an ideal addition to PDT regimens.

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1. Introduction

The subject of this project was the to study the use of photodynamic therapy (PDT) plus immunomodulation in the treatment of breast cancer, using a metastatic mouse model. The purpose of this proposal was to demonstrate the immune potentiation with a combinatory therapy between PDT and immunomodulators such as CpG oligodeoxynucleotides (ODN) and cyclophosphamide (CY). We were able to show that the use of PDT alone is temporally effective in the eradication of the primary tumor; that the use of either CpG ODN or CY in combination with PDT significantly potentiates the effectiveness of PDT based on the recurrence of the tumor and the survival of the animals.

2. Body

One in 8 women in the United States will develop breast cancer during her lifetime and 40,000 die each year. Deaths are due to tumors that have metastasized despite local control. Photodynamic therapy (PDT) is a promising cancer treatment in which a photosensitizer (PS) accumulates in tumors and is subsequently activated by visible light of an appropriate wavelength (1-3). The energy of the light is transferred to molecular oxygen to produce reactive oxygen species that produce cell death and tumor ablation. Mechanisms include direct cytotoxicity to tumor cells, shutting down of the tumor vasculature, and the induction of a host immune response. The precise mechanisms involved in the PDT-mediated induction of anti-tumor immunity are not yet understood. Among the potential contributing factors are alterations in the tumor microenvironment via stimulation of proinflammatory cytokines and direct effects of PDT on the tumor that increase immunogenicity.

Cyclophosphamide (CY) is a cytotoxic drug that damages DNA and kills cells but has historically also been used for immunomodulation (4). Low-dose intravenous CY can augment the humoral immune response and prolong survival in animal tumor models (5) and also has immunopotentiating effects in patients (6). CY has multifactorial mechanisms of immunopotentiation and selectively at low dose CY is specific for CD4+CD25+ T regulatory cells (Treg) (7, 8). CD4+CD25+ T cells represent approximately 5-10% of peripheral CD4+ T cells in mice and humans, and play a general role in immune regulation. Depletion of Treg cells by anti-CD25 mAb leads to increased tumor immunity (9); depletion of Treg cells might also be useful in vaccination, especially in the case of weakly immunogenic vaccines (10). We have previously shown that the combination of benzoporphyrin derivative-PDT and CY is highly effective in the treatment of a reticulum cell sarcoma in mice (11, 12), and we now wished to use this approach for the treatment a mouse model of metastatic breast cancer

The innate immune response is a first-line defense system in which individual Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular pattern (PAMP) molecules that are expressed by a diverse group of infectious microorganisms (13) and thereafter stimulate immune responses against a variety of pathogens .The extracts of the attenuated mycobacterium bacillus Calmette Guerin (BCG) have been used as a therapy for human bladder cancer (14). It was discovered that the active component of BCG was DNA with a potential to activate natural killer (NK) cells and

induce tumor regression in mice (15). Yamamoto et al sequenced mycobacterial genes and synthesized constituent oligodeoxynucleotides (ODN) (16); they concluded that certain self-complementary palindromes in these ODN were responsible for the immune stimulatory effects. The active palindromes contained at least one CpG dinucleotide and were more common in the genomes of bacteria compared to humans. Immunostimulatory sequences in bacterial (bDNA) that are structurally defined by their content of unmethylated CpG motifs (5'-purine-purine/T-CpG-pyrimidine-pyrimidine-3') are underrepresented in mammalian DNA (17). CpG DNA stimulates B cells, natural killer (NK) cells, dendritic cells (DC), and macrophages, regardless of whether the DNA is in the form of genomic bDNA or in the form of synthetic ODN (18). The level of immune stimulatory effects of an ODN depends to a great degree on the precise bases flanking the CpG dinucleotide (19). The resultant antigen-specific immunity is characterized by the production of high-affinity antibodies and the generation of cytotoxic T cells that provide long-lasting protection. TLR 9 recognizes d(CpG) dinucleotides present in specific sequence contexts (CpG motifs) in bacterial DNA, plasmid DNA, and synthetic oligodeoxynucleotides . TLR9 interacts with MyD88, which recruits IL-1 receptor associate kinase 4 (20). CpG DNA has strong stimulatory effects on murine and human lymphocytes in vitro and murine lymphocytes in vivo, such as: triggering B cell proliferation, release of IL-6 and IL-12; natural killer cell secretion of IFN-γ and increased lytic activity; and macrophage secretion of IFN-α/β, IL-6, IL-12, granulocyte-monocyte colony-stimulating factor, chemokines, and TNF-α.

We used 4T1 tumors growing in the mammary fat pad (orthotopic) in Balb/c mice that represents a model of stage III breast cancer. We used liposomal benzoporphyrin derivative mono-acid ring A, (BPD) as a PS and delivered light after 15 minutes for the PDT regimen and we tested PDT alone or in combination with two immunomodulation approaches. These consisted of low-dose CY administered 2 days before PDT, or CpG-ODN injected intratumorally three times before or after PDT. Our hypothesis was to allow the immune system to be more effective against the tumor by decreasing the effect of T regulatory cells or increase the effectiveness of the immune system by a multifunctional immunostimulant such as CpG-ODN.

4.1 MATERIALS AND METHODS

2.1.1. Animal model of metastatic breast cancer.

The Balb/c mammary adenocarcinoma 410.4 sub-line 4T1 cells were grown in RPMI 1640 media containing HEPES, glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. They were collected for injection by washing with PBS without Ca2+ and Mg2+, and adding trypsin-EDTA to the plate for 10 minutes at 37°C.

All animal experiments were carried out with the approval of the Subcommittee on Research Animal Care of Massachusetts General Hospital and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.160 Female Balb/c mice, weighing 20-25 g were depilated on one mammary fat pad. One million 4T1 cells were injected in one mammary fat pad suspended in 100 µL PBS. Tumors were grew as expected and reached a size of 5-6-mm diameter in 10-12 days after injection at which time they were used for PDT.

2.1.2. PS and light source for PDT.

BPD (liposomal benzoporphyrin derivative mono-acid ring A, Verteporfin for Injection, QLT Inc, Vancouver, BC, Canada) as a lyophilized powder, was reconstituted with 5% dextrose before injection. Mice were anesthetized with an i.p. injection of ketamine/xylazine cocktail (90mg/kg ketamine, 10 mg/kg xylazine).and BPD at 2 mg/kg was injected in 0.1 mL of PBS in the lateral tail vein.). Tails were warmed in water (50°C) to facilitate the procedure. PDT was carried out at 15 minutes after injection for BPD. Illumination was carried out using a 1W solid state diode laser (High Power Devices, Newark, DE) emitting light at 690-nm (+/- 2-nm) for activation of BPD. The laser was coupled into a 400-µm fiber via a SMA connector and light from the distal end of the fiber was focused into a uniform spot with an objective lens (No 774317, Olympus, Tokyo, Japan). The spot had a diameter of 1.2 cm and was positioned so that the entire tumor and a surrounding 2-3 mm of normal tissue were exposed to light. Mice were anesthetized as described above and the tumor bearing breast positioned under the spot. A total fluence of 150 J/cm2 was delivered at a fluence rate of 100 mW/cm2. At the completion of the illumination mice were allowed to recover in an animal warmer until they resumed their normal activity.

2.1.3. Immunostimulants

Some groups of mice received a single IP injection of CY at a dose of 50 mg/kg (dissolved in PBS) and they received PDT 48 hours later. Control groups received CY alone and PDT alone.

CpG ODN and control non CpG ODN were obtained from Coley Pharmaceutical Group (Wellesley, MA); these ODN consisting of 20 bases and have two CpG motifs. The ODN used was: 1826 Class B. Sequence. A non- CpG Control ODN was, 2138 Class B (control). ODN concentrations in the final solution were 10 µg/100µl and 20 µg per mouse was injected peritumorally. After the tumor reached 5 mm diameter two possible treatments were done: CpG-ODN administered in 3 doses (Day 1, 4, 7) and PDT carried out on day 7. The second one was, PDT day 1 and CpG-ODN on days, 1, 4, and 7. The total dose of CpG ODN per mouse was 60 µg.

2.1.4. Animal follow-up.

Mice were examined and weighed three times a week. Two orthogonal tumor dimensions (a and b) were measured with vernier calipers and the volume calculated according to the formula = $4/3\ \pi\ [(a+b)/2]^3$. Mice would be considered cured when the tumor did not return after 90 days. Mice were sacrificed according to protocol when their primary tumors reached a volume of 200 mm^3 or when they reached a moribund state (loss of >15% body weight) due to uncontrolled progression of metastatic disease.

2.1.5. Data analysis and statistics.

All values are expressed as $\pm$ standard error of the mean. Comparison between two means was carried out using the Mann-Whitney U-test. Survival analysis was performed using the Kaplan-Meier method. Survival curves were compared, and differences in survival were tested for significance using a log rank test in the computer program GraphPad Prism (GraphPad Software Inc., San Diego, CA). The tumor growth curves were analyzed by transforming the data to a logarithmic scale and comparing the slopes. P values of < 0.05 were considered significant.

2.2. RESULTS

2.2.1. Model of metastatic breast cancer in Balb/c mice.

The 4T1 mammary carcinoma tumor is a poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes to lymph nodes, lung, liver, brain, and bone and is disseminated via the blood stream following growth of the primary tumor in the female mouse mammary gland (5, 21). This disease progression closely parallels human breast cancer and makes the 4T1 tumor an excellent model for human disease (22) and a rigorous animal model of advanced spontaneous metastatic disease.

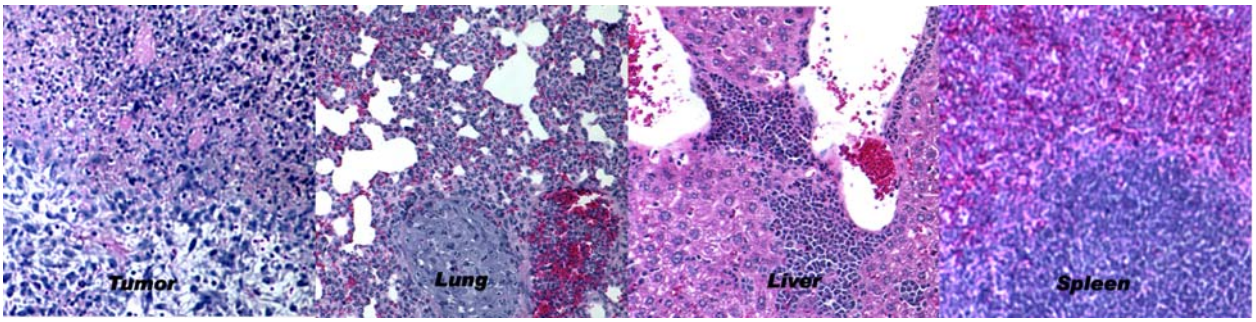

Figure 1. H&E stained paraffin fixed sections of tissues from mice with 4T1 tumors. There are metastases after twenty days, in the liver, spleen, and lung.

2.2.2. PDT alone and combination of PDT and low-dose CY.

Ten mice were treated with BPD-PDT, (2 mg/kg IV followed after 15 minutes by 150 J/cm^2 690-nm light delivered at 100 mW/cm^2) carried out 8 or 9 days after the tumor was implanted. Another ten mice received the same regimen of BPD-PDT delivered two days after a single-low dose of intraperitoneal CY. The tumor growth curves are shown in Fig 2A and the corresponding survival curves in Fig 2B of these groups of mice compared to 10 control 4T1 tumor-bearing mice. PDT alone had remarkably little effect on these 4T1 tumors. There was a black eschar formed in the center of the tumor the next day as expected with the vascular regimen of light delivered 15 minutes after BPD injection. However the tumor growth was only transiently interrupted and although the tumor growth curves are significantly different, all mice were sacrificed mostly due to progressive tumor but some due to metastatic disease between 20 and 35 days after tumor implantation. The mice that received the combination of BPD-PDT and low dose CY achieved a significant period of tumor stabilization between PDT and approximately day 32 after tumor implantation, after which time the tumors grew slowly (Fig 2A). Most of these mice were sacrificed due to progressive metastatic disease (i.e. mice descended into a moribund state) rather than due to uncontrolled growth of the primary tumor. The Kaplan-Meier survival curves are shown in Figure 2B. The median survival times of the groups of mice are as follows: no treatment control 24 days, BPD-PDT alone 35 days ($P<0.01$) and BPD-PDT + CY 47 days ($P<0.0005$ vs control; $P<0.005$ vs PDT alone).

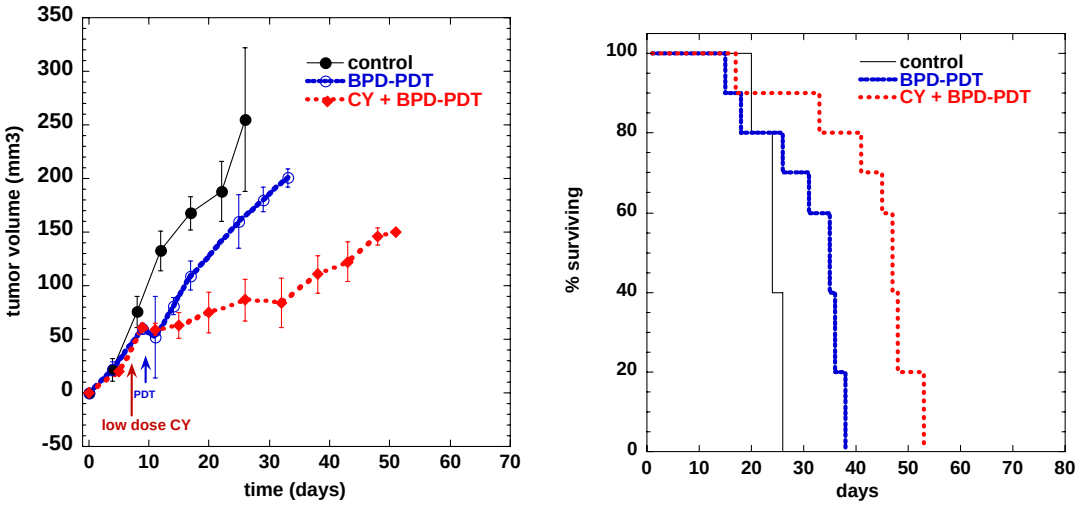

Figure 2. A. Tumor growth curves of mice with 4T1 tumors receiving either; no treatment, BPD-PDT alone or BPD-PDT 2 days after low-dose CY. All mice challenged with 4T1 tumors die between 20 to 26 days after the procedure is done. Compared with control the treatment with BPD-PDT, only delays the progression of the tumor, but with CY and PDT the mean tumor growth rate is significantly slower. B. Kaplan-Meier curves of mouse survival of the treatment groups described in Fig 2A. Mice treated with BPD-PDT have longer survival (median 35 days) than control mice (24 days; P<0.01), but adding CY to this treatment does doubles the survival time (47 days, P<0.0005 vs control; P<0.005 vs PDT alone).

2.2.3. Combination of PDT and intratumoral CpG oligonucleotides.

Mice with 4T1 tumors at day seven were distributed in five groups, (i) control no treatment, (ii) a group treated with BPD-PDT alone, (iii) a group treated with CpG alone, injected in three doses of 20ug (200ul) distributed in different intradermal points around the site where the tumor was implanted, counting first day as the day of treatment, CpG was injected: day 1, 4 and 7. The last two groups were treated with a combination of BPD-PDT and CpG, (iv) a group of mice was treated the first day with BPD-PDT and subsequently CpG was injected at day 1, 4, and 7; (v) a group treated with CpG on day 1, 4, and 7 and BPD-PDT carried out on day 7. The results in terms of tumor growth curves are shown in Fig 3A and the survival curves are given in Fig 3B. The median survival times for groups involving CpG were CpG alone 32 days; PDT + CpG 53 days; CpG + PDT 55 days (P for both orders vs PDT alone < 0.0001; and P for both orders vs CpG alone <0.0001).

The results in this series of experiments show that combination of BPD-PDT and CpG immunostimulation regardless of order of administration is significantly better than either treatment alone both in suppressing the local tumor growth and extending the survival. CpG alone actually appeared to be worse than no treatment in terms of local tumor growth (Fig 3A) but significantly better (P < 0.005) than no treatment in prolonging survival (Fig 3B) presumably due to a weak antimetastatic effect.

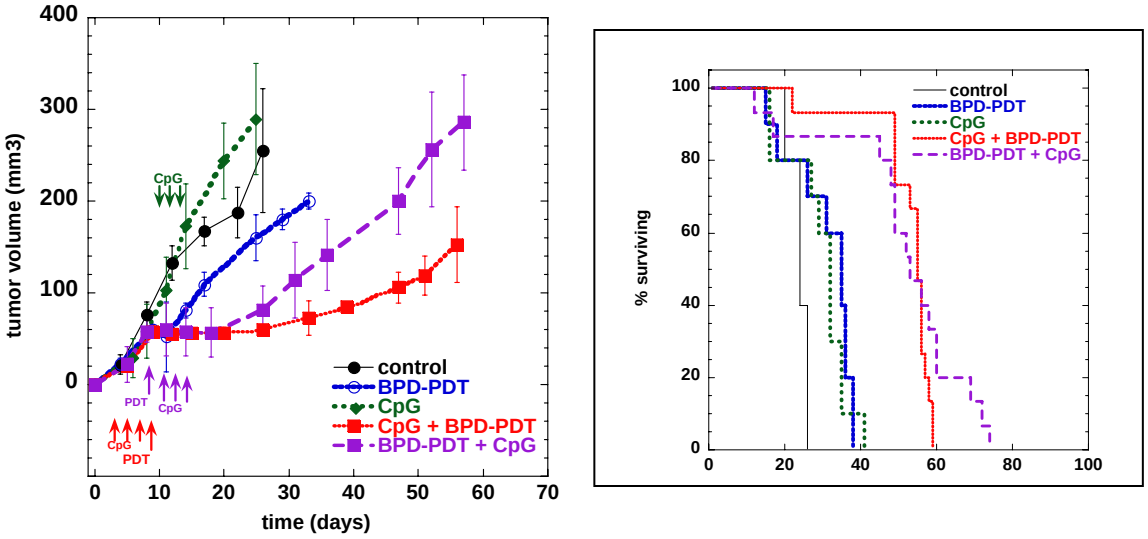

Figure 3. A. Tumor growth curves of mice with 4T1 tumors receiving either; no treatment, BPD-PDT alone, CpG alone in 3 injections, or CpG combined with BPD-PDT either before or after. B. Kaplan-Meier curves of mouse survival of the treatment groups described in Fig 2A. Survival times were (CpG alone median 32 days); PDT + CpG 53 days; CpG + PDT 55 days (P for both orders vs PDT alone < 0.0001; and P for both orders vs CpG alone <0.0001).

3. DISCUSSION

BPD-PDT only slightly delayed the progression of 4T1 tumors. It is known that BPD is a vascular photosensitizer, and can destroy the tumor microcirculation therefore leading to apoptosis and necrosis of the tumor cells. PDT can increase the expression of antigens in the dying tumor, with the possibility that antigen-presenting cells could recognize these tumor antigens and consequently increase the immune response towards the local tumor and/or distant metastases. However the relative lack of major tumor destruction by PDT alone in this model of orthotopic mammary fat-pad 4T1 tumors makes this mechanism less likely. This model of breast cancer is stage III, at the time of the treatment the tumor has already metastasized and therefore an approach is needed that has both a local effect and a distant or systemic effect. Therefore we decided to combine PDT with immune stimulation regimens.

The pathophysiology of cancer is very complex and tumors have been proposed to develop multiple mechanisms to avoid the host immune response (23, 24). One of the most important of these immune evasion mechanisms described in the literature is the induction of T-regulatory cells (25), a group of cells capable of suppressing the tumoricidal activity of both CD4 and CD8 cell, either by direct contact or through the secretion of immunosuppressive cytokines such as TGF-β and IL-10 (26). The use of low dose CY has shown certain selectivity against CD4+CD25+ T regulatory cells (27). Previous studies from our laboratory (11) have shown that certain tumors lead to an increase in T regulatory cells in splenocyte suspensions from tumor-bearing mice as measured by flow cytometry, and that low dose CY can deplete a certain proportion of these T regulatory cells. Whereas we have seen large effects leading to complete cures

and induction of long-term memory anti-tumor immunity by combining CY with BPD-PDT in J774 tumors (12), in the case of 4T1 tumors the effect was more subtle. This reduction of efficacy of the combination was probably due to the lower effect of PDT alone. PDT can effect an initial ablation of J774 tumors while it had much less effect on the small 4T1 tumors. However the combination of BPD-PDT and CY was significantly better than either treatment alone, showing that the immune system can generate a more vigorous response when the T regulatory cells are depleted.

The use of CpG-ODN in combination with BPD-PDT had a bigger impact in the treatment of this model of breast cancer. There was a significant delay in the local tumor progression and a corresponding increasing in the survival of the mice. One important question to answer in designing any combinatory therapy is the order of administration of the respective component treatments. We had supposed that a chief role of CpG ODN is to attract host immune cells such as dendritic cells, natural killer cells and macrophages, into the tumor and surrounding tissue (28). Therefore CpG ODN should perform better if administered before PDT rather than afterwards, because PDT can shut down the blood vessels thus preventing leukocyte access into the tumor after PDT. However if the chief role of CpG ODN is to potentiate the phagocytosis of necrotic or apoptotic tumor cells by already present dendritic cells and to induce dendritic cell maturation and migration to draining lymph nodes (29), then the reverse order (i.e. CpG ODN after PDT) may be superior because the PDT induced damage will be there for the DCs to take up when stimulated with CpG. The results showed that both orders of administration were significantly better than either treatment alone. CpG first and PDT afterwards gave a somewhat better control of local tumor growth but somewhat less of a survival advantage, compared to PDT first and CpG afterwards where the control of the local tumor was not so good but the survival was slightly better. This may suggest that the local inflammation caused by intratumoral injection of CpG could potentiate the effect of PDT on the primary tumor (perhaps by increasing the amount of BPD accumulating in the tumor due to increased microvascular permeability). PDT first and CpG later could produce a better systemic immune response due to the creation of potential tumor antigenic fragments before the immune stimulation led to their take up by antigen presenting cells.

Our future work will focus on defining the specificity of low-dose CY on T regulatory cells using monoclonal antibodies against some specific cell markers to analyze the effect on different classes of lymphocytes and to test the anti-CD25 serum as an alternative means to deplete T-regulatory cells in tumor bearing mice before PDT. In order to understand the synergistic combination of PDT and CpG we need to test the non-CpG ODN as a negative control and further explore the optimum order of administering the two treatments. In the future since this model of 4T1 tumor has so far proved resistant to complete cure we will investigate the triple combination of PDT, low-dose CY and CpG.

4. Conclusions

PDT is an alternative therapeutic approach for some type of cancer, and in this research we have demonstrated that certain immunomodulatory therapies significantly potentiate the effect of PDT based on survival and tumor size, in this murine stage III model of breast cancer. Complete optimization of the process of induction of anti-tumor immune response by PDT and adjuvant treatments requires further work including testing of triple therapy (PDT + CpG + CY).

5. Key Research Accomplishments

- BPD-PDT delayed the progression of 4T1 tumors compared with the control
- BPD-PDT and CY was significantly better than either treatment alone,
- The use of CpG-ODN in combination with BPD-PDT had a bigger impact in the treatment of this model of breast cancer. There was a significant delay in the local tumor progression and a corresponding increase of the survival of the mice.

6. Reportable Outcomes

Scientific abstract, Era of Hope 2005 DoD Breast Cancer Research Program Meeting Pennsylvania Convention Center, Philadelphia, Pennsylvania, June 8-11, 2005.

Abstract presentation. AACR meeting. Washington, DC. April 1-5 2006

Castano AP and Hamblin MR. Enhancing photodynamic therapy of a metastatic mouse breast cancer by immune stimulation. In: Chen WR, Editor. Biophotonics and Immune Responses. Jan 20-25 2006, Bellingham, WA, The International Society for Optical Engineering, Proc SPIE 6087. in press.

Hamblin MR, Castano AP and Mroz P. Combination immunotherapy and photodynamic therapy for cancer. In: Chen WR, Editor. Biophotonics and Immune Responses. Jan 20-25 2006, Bellingham, WA, The International Society for Optical Engineering, Proc SPIE 6087. in press.

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